

Original Article

Single administrations of high and low doses of acetaminophen causes different effects on COX-2 gene expression and on tissue damage in liver and kidneys

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Abstract

Introduction: High dose of acetaminophen (APAP) is known to have hepatotoxic and nephrotoxic effects and studies show that these toxicities are dependent on the function of phase I bioactivation enzymes- Cyp450- and phase II biotransformation enzymes especially glucuronosylation and sulfonation pathways. However, the role of cyclooxygenase (COX) as an inflammatory mediator in toxic effects of APAP has not been explained satisfactorily yet.

Methods: In this study, we aimed to find out if there is any association between APAP hepatotoxicity and COX-2 expression at mRNA levels. Male Balb/C mice were treated with a single high dose (300 mg/kg BW) or low dose (30 mg/kg BW) of APAP.

Results: Following APAP treatment, serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes were measured as the biochemical markers of hepatocellular damage. Then liver and kidney biopsies were processed and examined for histopathological changes as well as for total RNA extraction and COX-2 gene expression. Serum ALT/AST levels were significantly ($P < 0.05$) higher and there were hepatotoxic damages after 24 hours in mice exposed to high dose of APAP (300 mg/kg BW). However, no obvious nephrotoxicity was observed in mice treated with either low or high doses of APAP. Based on RT-PCR data, the COX-2 specific mRNA was not expressed in liver tissues of either control or APAP-treated mice, while, it was expressed in kidney tissues of both control and APAP-treated mice.

Conclusion: These data may suggest that unlike in liver, COX-2 expression in kidney may play a protective role in APAP-related hepatotoxicity.

Keywords:

Acetaminophen (APAP);
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Introduction

Acetaminophen (APAP) is one of the most widely used antipyretic and analgesic drugs world-wide (Hinz and Brune, 2012). APAP works by lowering

cyclooxygenase (COX) products, prostaglandin synthesis and affects central and/or peripheral nervous system (Warner and Mitchell, 2004). However, it is only a weak inhibitor of COX and appears to have very little anti-inflammatory activity (Botting, 2000a; Warner and Mitchell, 2004).

Cyclooxygenase exists principally in three active isoforms: cyclooxygenase-1 as a constituent isoform, cyclooxygenase-2 as an inducible isoform and a third form, cyclooxygenase-3 that has been reported in some species (Botting and Ayoub, 2005; Lucas et al., 2005). APAP hepatotoxicity is the most common cause of death due to acute liver injury in the developed world (Bessems and Vermeulen, 2001; Imaeda et al., 2009). The work on the APAP metabolism demonstrated that pharmacokinetics and toxicity of APAP rely on the activities of phase I enzyme bioactivation- Cyp450- and phase II enzyme biotransformation especially glucuronidation and sulfation. In the phase I, APAP is converted to a quinoneimine, namely N-acetyl-para-benzoquinoneimine (NAPQI), a cytotoxic electrophile that binds to cellular proteins and induces hepatotoxicity (Bessems and Vermeulen, 2001). The initial event in APAP hepatotoxicity is centrilobular necrosis and apoptosis as observed by the release of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes to serum (Bessems and Vermeulen, 2001; Rowden et al., 2005). This can lead to secondary activation of the innate immune response involving up-regulation of inflammatory cytokines with activation of NK cells, NKT cells and neutrophils (Liu et al., 2004; 2006). In turn, expression of hepatic stress and detoxification genes are induced in response to chemical injury while anti-inflammatory cytokines block this process or at least suppress the intensity of the cascade. Therefore, the equilibrium between the effects of proinflammatory and anti-inflammatory cytokines is thought to determine the outcome of injury, whether in the short term or long term (Aleksunes et al., 2005; Dinarello, 2000).

Materials and methods

Animals

Male Balb/C mice weighing 25 ± 2 g and 8-10 weeks old were purchased from the Pasteur Institute of Iran and housed in polycarbonate cages containing hardwood chip bedding at standard conditions. Twenty four mice were randomly divided into six groups and each group ($n=4$ /group) was kept together in a separate cage. The temperature of the animal room was adjusted to 22 ± 2 °C with a 12-h light/dark cycle. The animal studies were approved by

the Medical Ethics Committee of Tarbiat Modares University.

Induction of APAP toxicity

Control groups (6 and 24 hours); each mice received 500 μ l of PBS alone. Low dose APAP treated groups; injected a single intraperitoneal (ip) dose of APAP (30 mg/kg BW, dissolved in 500 μ l PBS). High dose APAP treated groups; injected single ip dose of APAP (300 mg/kg BW, dissolved in 500 μ l PBS).

Enzyme activity assays

Blood was collected immediately from each group at 6 and 24 hours after treatment. Sera were separated by centrifugation at $1500 \times g$ for 10 min at 4 °C. The activities of serum ALT and AST as the biochemical markers of hepatocellular damage, were measured using commercial kits purchased from Parsazmun, Iran according to the manufacturer's instructions. The absorbances were measured at 340nm.

Histology

For histopathological study, the liver and kidney of each mouse were removed. Each tissue was divided into two parts. One part was fixed by immersion in 10% neutral buffered formalin then embedded in paraffin, cut in to 5 μ m sections and stained with hematoxylin and eosin for light microscopic examination. A histopathologist evaluated all liver and kidney sections and recorded the observations. Another part of each tissue was stored in -80°C until the day of COX-2 gene expression analyses. The APAP-treated mice groups that showed definite histopathological abnormalities in the liver (hepatic necrosis) were selected for comparison with non-treated mice.

Gene expression analysis

Total tissue RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. RNA pellets were resuspended in diethyl/pyrocarbonate-treated deionized water. RNA samples were analyzed by agarose gel electrophoresis and integrity was confirmed by visualization of intact 18S and 28S rRNA under UV light. Spectrophotometric study (NanoDrop, Thermo Scientific 2000c) was confirmed the purity of total RNA and then its concentration was determined. The 1-2 μ g RNA was reverse-transcribed to cDNA at 42°C

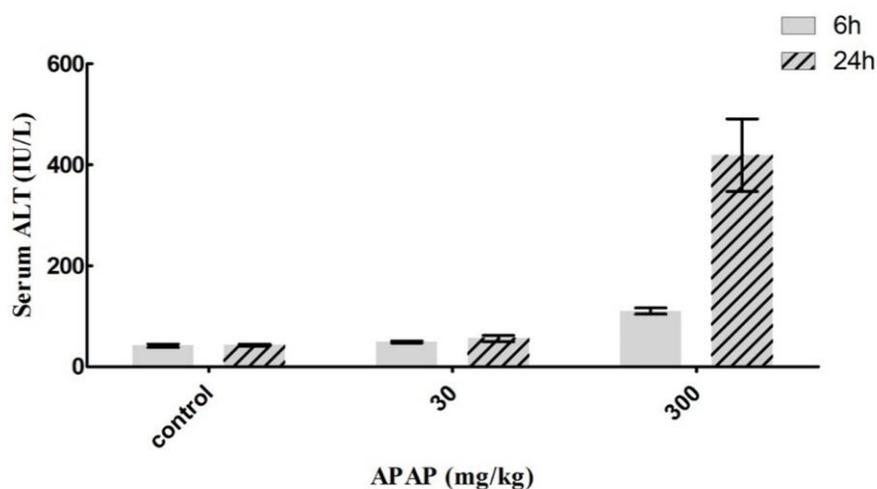


Fig.1. Effect of low and high dose acetaminophen on serum ALT in mice. Mice (n=4) treated with a single low dose (30 mg/kg BW) or high dose APAP (300 mg/kg BW). Control group (n=4) received vehicle alone. Blood was collected and serum samples were analyzed for ALT level. The results are mean \pm SD of 4 samples. $P < 0.05$ is considered significant compared to control group.

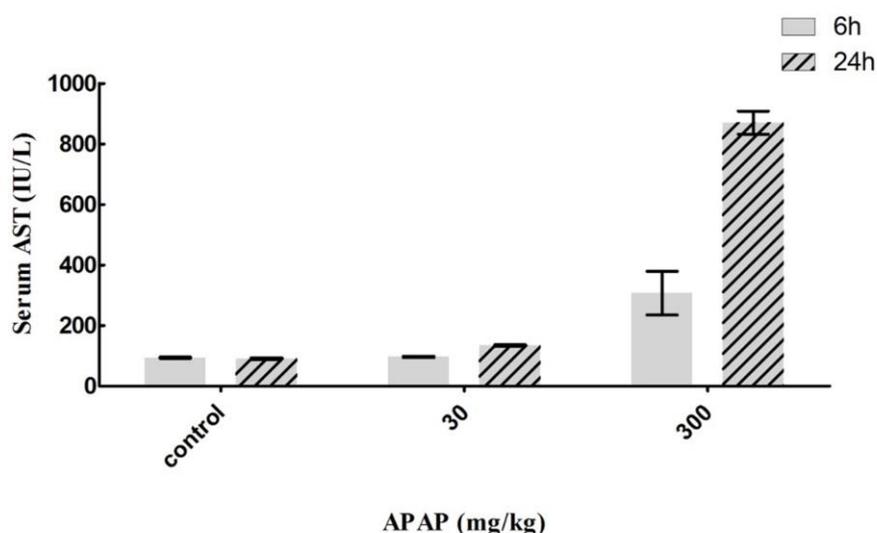


Fig.2. Changes in serum AST level in mice treated with a toxic dose of acetaminophen. Mice (n=4) were administrated either low dose of APAP (30 mg/kg BW) or high dose APAP (300 mg/kg BW). Control group (n=4) received vehicle alone. Blood was collected and serum ALT concentration was determined. The results are mean \pm SD of 4 samples. $P < 0.05$ is considered significant compared to control group.

for 60 min. After enzyme inactivation (95°C, 10 min) cDNA fragments were amplified for 35 cycles using gene-specific primers for COX-2 (sense: TGTGACTGTACCCGGACAGG; antisense: TGCACATTGTAAGTAGGTGGAC) and housekeeping gene, hypoxanthine guanine phosphoribosyl transferase (HPRT) (sense: TCAACGGGGACATAAAA; antisense: TAACCAGGGAAAGCAAAG). PCR products were resolved on 2% agarose gels and visualized using Uvitec gel documentation systems (Uvitec ArminTeb, Iran). COX-2 gene expression study was performed

by statistical analysis using Uvitec FireReader Software (Cambridge) to compare the groups.

Results

Effects of APAP treatment on enzyme release

Administration of a single low dose of APAP (30 mg/kg BW) to mice caused no significant changes in serum ALT/AST levels compared to controls. However mice injected with a single high dose of APAP (300 mg/kg BW) and sacrificed 6h or 24h after treatment showed a significant ($P < 0.05$) increase in

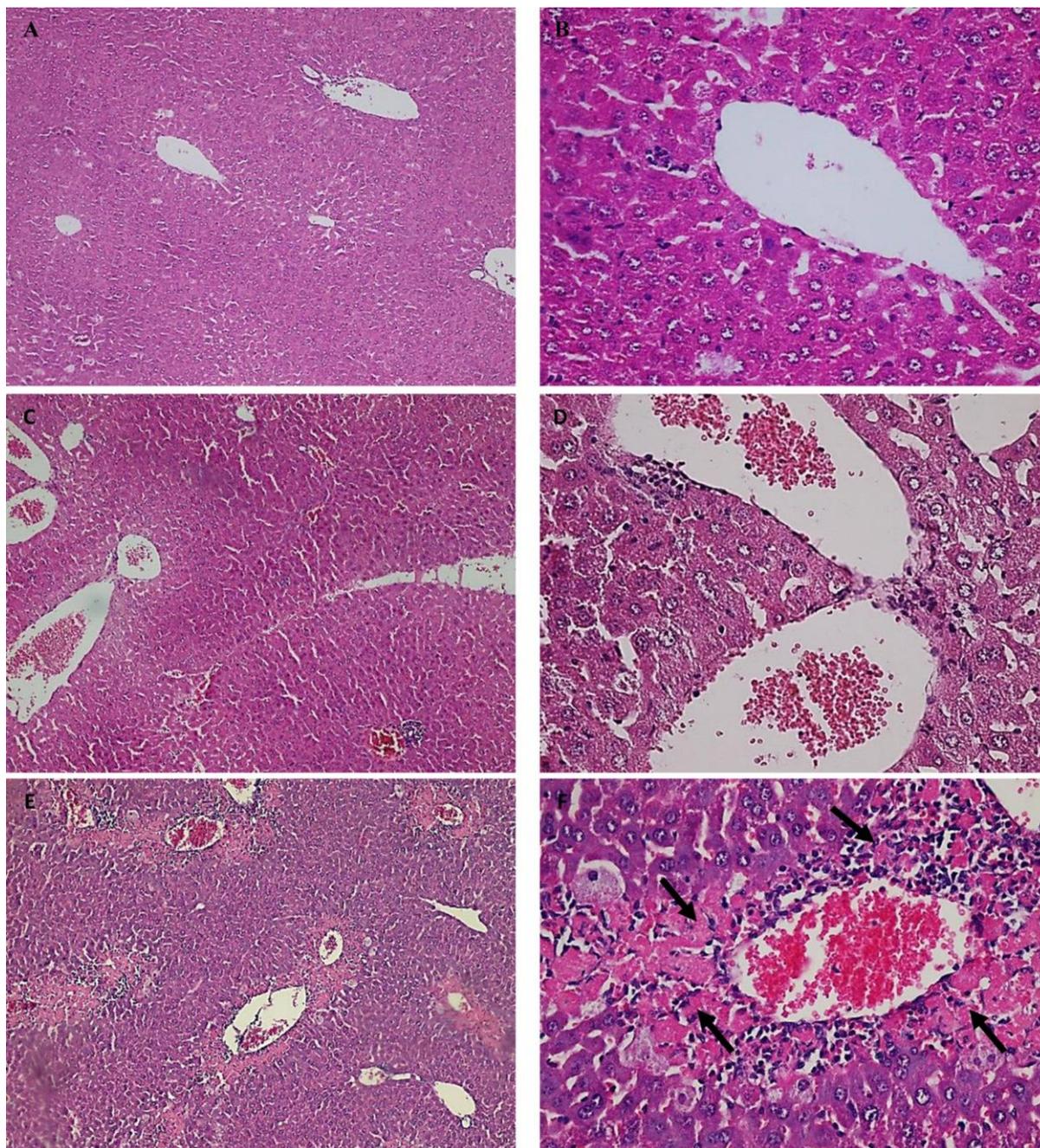


Fig.3. Histopathological changes in liver tissue of mice treated with acetaminophen. Mice were treated with an acute dose of APAP (300 mg/kg BW) and liver biopsies were processed for H&E staining. Mice were sacrificed after 6 or 24 hours for histopathological examinations and the results are recorded. Sections A and B, show liver histology of control (untreated) mice. Sections C and D, liver histology of mice treated for 6 hours. Sections E and F show liver histology of mouse sacrificed after 24 h of APAP treatment. Arrows indicate centrilobular necrosis. Magnification of figure A, C and E is 10X and for sections B, D and F is 40X.

serum levels of ALT and AST. There was approximately 3-folds increase in the enzyme markers 6h after APAP injection which was further increased (~10 fold) in mice sacrificed after 24 hours of treatment (Fig. 1 and 2).

Histopathological changes

Liver and kidney tissue biopsies obtained from mice treated with a high dose APAP (300 mg/kg BW) for 6

and 24 hours were processed and examined for histological changes. Treatment of mice with a high dose APAP (300 mg/kg BW) caused an extent of hepatic necrosis in the centrilobular region of the liver at 24h time point (Fig. 3E and F). However, in mice sacrificed 6 hours after APAP intoxication, the histological changes in the liver tissue was limited to inflammation (Fig. 3C and D) compared to the control group (Fig. 3A and B). The results of APAP-induce

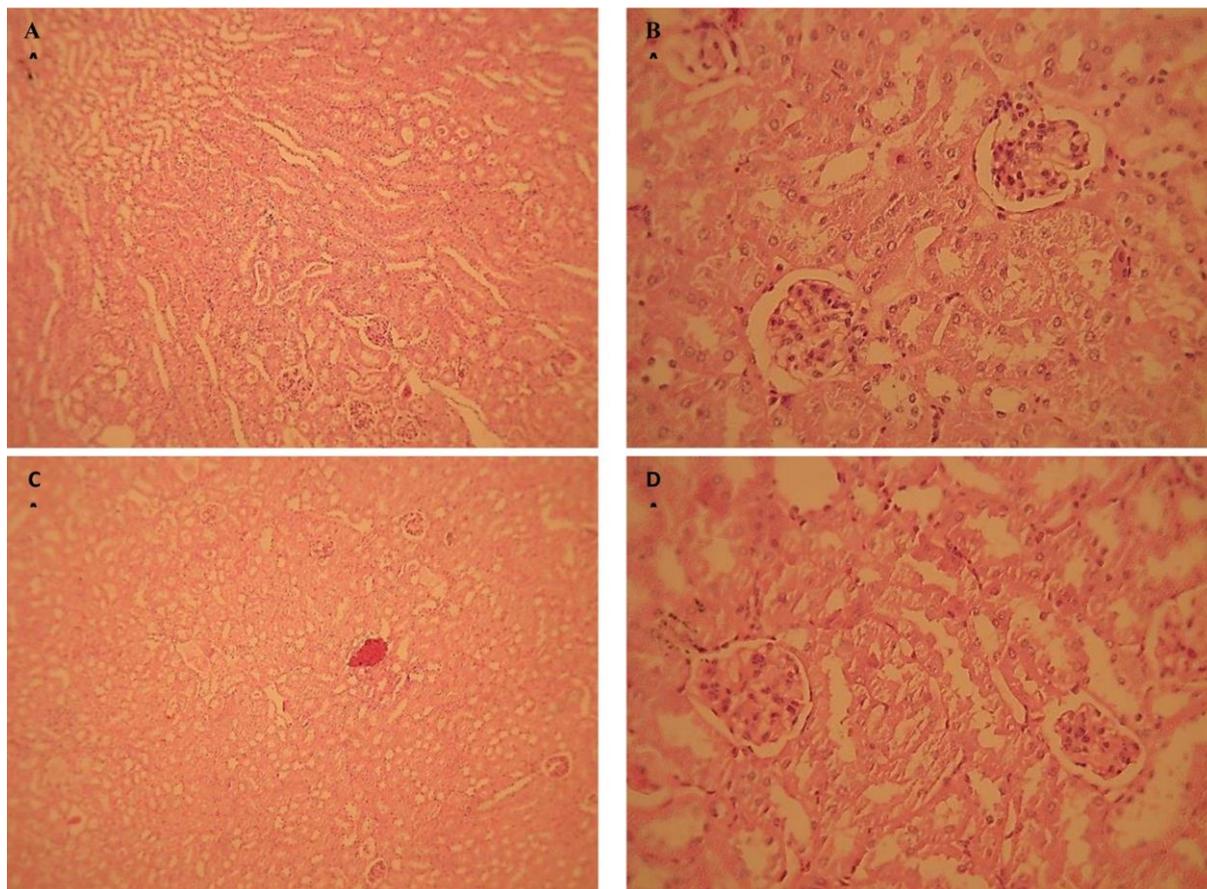


Fig.4. Effects of high dose acetaminophen treatment on mice kidney histology. Section A and B, kidney tissues of untreated mouse (control). Sections C and D, kidney tissues of mouse 24h after treatment. Magnification of A, C and B, D is 10X and 40X respectively. Mice were treated with an acute dose of APAP (300 mg/kg BW) and kidney biopsies were processed for H&E staining. Mice were sacrificed after 6 or 24 hours for histopathological examinations and the results are recorded.

liver injury on polymorphonuclear (PMN) infiltration and histologic changes summarized in Table 1. No significant liver damage was observed in mice treated with a low dose APAP (30 mg/kg BW). For all samples, histopathological stage was zero (data not shown). Histological evaluation of kidney sections prepared from mice treated with high dose of APAP (300 mg/kg BW) for 24 hours showed no significant pathological changes when compared to respective controls (Fig. 4).

COX-2 gene expression profiles

The results of reverse transcriptase PCR (RT-PCR) performed on RNA samples isolated from liver and kidneys of APAP-treated mice showed that COX-2 specific mRNA was not detectable in samples prepared from liver tissues. COX-2 specific mRNA was absent in liver samples from normal and APAP-treated mice. However, the samples prepared from

kidney tissues were positive in COX-2 expression. There was no significant difference in COX-2 gene expression. The level of COX-2 expression was comparable in kidney samples prepared from both control groups and those treated for 24 hours with a high dose APAP (300 mg/kg BW) (Fig. 5).

Discussion

The aim of this study was to understand the role of COX-2 in APAP-induced hepatotoxicity and nephrotoxicity, because the hepatoprotective role of COX-2 is still being debated. In the present study COX-2 expression at mRNA levels was compared in liver and kidney tissues following APAP-treatments. APAP is an over the counter drug used as an analgesic and antipyretic drug. The liver is the primary site in the body where APAP is metabolized. In hepatocytes, APAP first undergoes sulphonation

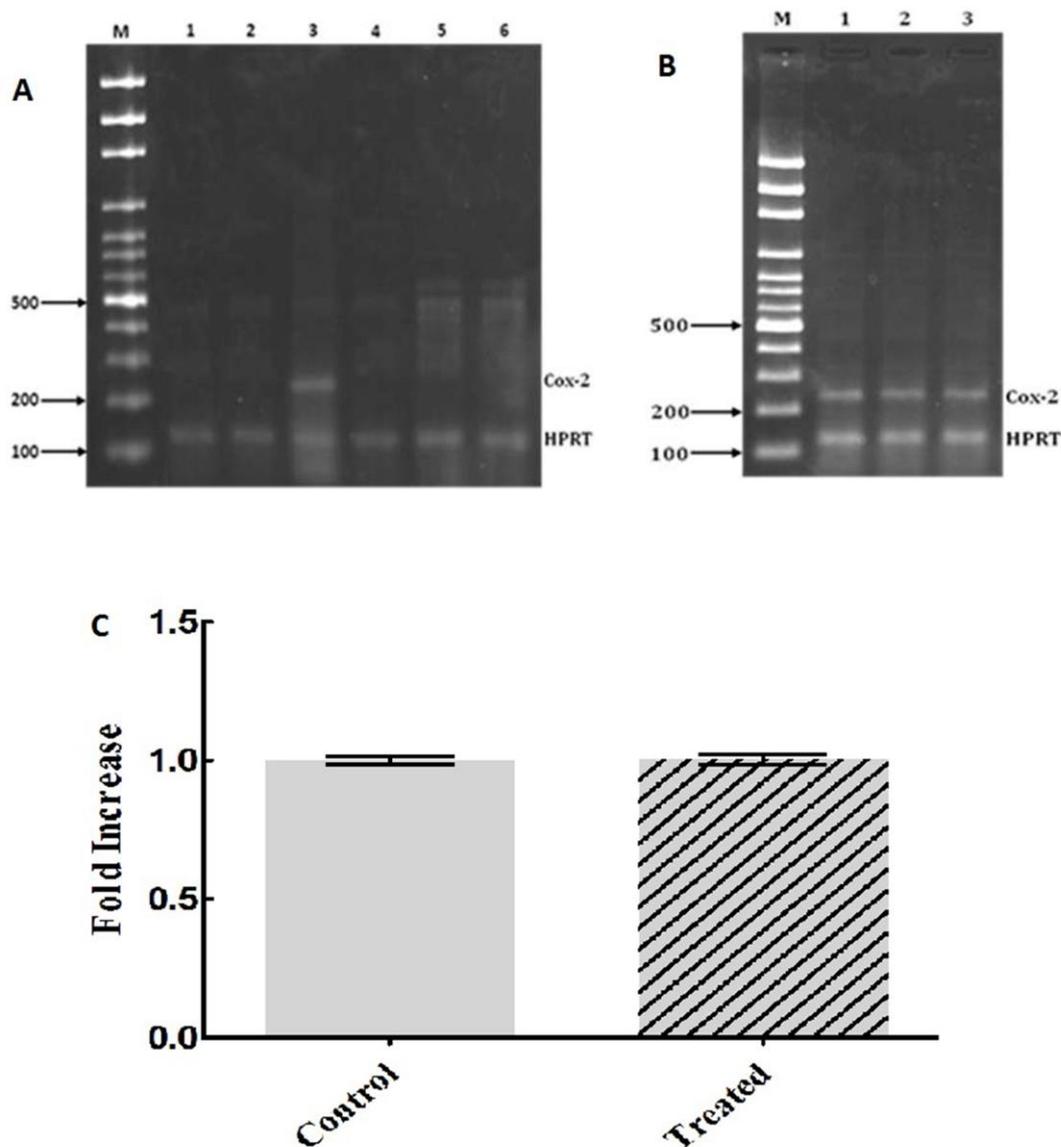


Fig.5. Determination of COX-2 expression using reverse transcriptase PCR (RT-PCR) in mice liver and kidney tissues after acute acetaminophen treatment. Mice were treated with a single high dose APAP-dose (300 mg/kg BW) and sacrificed after 24 hours. **Section A** shows liver samples prepared from mice 6h after APAP treatment (Lanes 1, 2) and lanes 4 and 5 show samples obtained 24h after APAP treatment. Lane 6, is normal sample prepared from untreated animal. Lane3 represent normal kidney tissue as the positive tissue control expressing COX-2. **Section B**, shows COX-2 expression in kidney tissues is presented in line 1 (normal sample) used as untreated kidney tissue (control). Lanes 2 and 3 show kidney samples from mice treated for 6 and 24h respectively. Lane M is marker. HPRT gene a house keeping gene was used as control. **Section C**, compares fold changes in COX-2 mRNA expression in kidney tissues by statistical analysis.

(conjugation to sulphate) and then glucuronidation (APAP- glucuronide conjugation) which facilitate detoxification of the drug from the body. An excessive dose of APAP in the liver can saturate the conjugation pathways (sulphation and glucuronidation) as a result of which the drug is processed through the cytochrome P-450 system. The P-450-mediated APAP biotransformation forms an intermediate metabolite known as NAPQI, which turns out to be a toxic compound (Hinz et al., 2008).

APAP reduces levels of prostaglandin metabolites in urine but does not reduce synthesis of prostaglandins by blood platelets. It is a weak inhibitor *in vitro* of both COX-1 and COX-2, the possibility exists that it inhibits a so far unidentified form of COX, perhaps COX-3. In animal studies, COX enzymes in homogenates of different tissues vary in sensitivity to the inhibitory action of acetaminophen. This may be evidence that there are more than two isoforms of the enzyme. Recently, a variant of COX-2 induced with

Table 1: Effect of acetaminophen on polymorphonuclear (PMN) infiltration and histologic changes of liver injury in treated mice. The mean number of neutrophils was determined by counting the numbers of polymorphonuclear inflammatory cells (neutrophils) in 10 randomly selected high power fields (400x). Results are mean \pm SD of 4 samples. Histological changes were graded based on findings of necrosis and congestion 6h and 24h after treatment by toxic dose of acetaminophen. Scoring system included: (-) for none (no change), (+) for mild changes, (++) for moderate changes and (+++) for severe changes.

Groups	Number of infiltrated neutrophils (PMN)	Congestion	Focal necrosis	Liver histopathology	Grading
Control	1.4 \pm 0.89	-	-	Normal	0
Acetaminophen (300mg/6h)	3.8 \pm 0.84	+	++	Pathologic changes such as: infiltration of PMN, focal necrosis of hepatocytes, congestion and kupffer cell hyperplasia	1
Acetaminophen (300mg/24h)	26.3 \pm 4.4	++	+++	Sever pathologic changes such as: infiltration of PMN , nomerous focal necrosis of hepatocytes, congestion and kupffer cell hyperplasia	2

high concentrations of nonsteroidal anti-inflammatory drugs was shown to be highly sensitive to inhibition by APAP. Therefore COX-3 may be a product of the same gene that encodes COX-2, but have different molecular characteristics (Botting, 2000b).

The hepatotoxicity and nephrotoxicity of APAP has been reported from different laboratories (Bessemers and Vermeulen, 2001; Jaeschke et al., 2011). Our experience shows low dose and high dose APAP injected to mice caused differential effects in biochemical and histopathological parameters in liver and kidneys. These data further confirm that APAP at a toxic dose changes plasma concentration of serum ALT and AST which indicate liver cell injuries. These data were corroborated with histopathological changes in liver and kidney tissues. These results are inconsistent with our previous reports on APAP-induced hepatotoxicity *in vivo*, suggesting that these APAP-related toxicity is limited to high dose treatments (Allameh et al., 1997). In contrast, low dose APAP treatments (30 mg/kg BW) showed no significant changes in serum ALT and AST levels that was in agreement with previous reports by Jeong and co-workers (2005).

The data obtained from gene expression analysis

shows that COX-2 specific mRNA was not detected in mice liver, and there was no further changes in COX-2 expression in response to high dose APAP (300 mg/kg BW) administered for 24 h to mice. Absence of detectable levels of COX-2 in liver implies that COX-2 is not necessarily involved in APAP-induced toxicity and related adaptive responses. Hence, the data obtained from COX-2 analysis in liver tissue are insufficient to establish a relationship between APAP toxicity and COX-2 expression. However, COX-2-related mRNA was expressed in kidney tissue in both APAP-treated and untreated groups of mice. Although there was no significant difference in COX-2 expression between APAP-treated and controls, APAP intoxication failed to alter COX-2 expression in kidney tissues. Interestingly, there was no significant histological changes in kidney tissues of mice treated with APAP, however, COX-2 expression was detected in this tissue. Despite the expression of COX-2 in kidney tissue, there are no convincing evidences to show involvement of this enzyme is nephrotoxicity.

Comparison of COX-2 expression in liver and kidneys may suggest that COX-2 and downstream pathways play a role in APAP-induced nephrotoxicity. In this

context, an inducible isoform of COX i.e. COX-4 has been characterized in isolated cells which may also mediate some of the actions of APAP (Botting and Ayoub, 2005).

The contribution of COX-2 in proinflammatory and anti-inflammatory pathways is unclear. COX-2 is widely accepted to be the inducible form with a role in inflammatory processes (Rouzer and Marnett, 2009). Although therapeutic dose of APAP has anti-inflammatory effects as a COX-2 inhibitor (Lee et al., 2007; Lucas et al., 2005), in drug-induced liver injury by APAP in C57B1/6 mice, a protective role of COX-2 has been reported (Reilly et al., 2001).

COX-1 and COX-2 enzymes carry out essentially the same catalytic reaction and have similar primary protein structures, but the regions regulating gene expression of COX enzymes show little similarity. In addition, COX-1 specific mRNA is relatively stable while COX-2 mRNA is an immediate early gene that is activated by a wide variety of hormones, growth factors, inflammatory factors and cytokines (Dubois et al., 1998). Tumor necrosis factor (TNF) as a proinflammatory cytokine induces COX-2 gene expression while TNF receptors has been down regulated due to APAP toxicity (Jeong et al., 2005).

The high degree of variability in APAP toxicity was observed due to a variety of factors including strain, age and gender. The intra-strain variability in toxicity has been reported due to differences in epigenetic effects and differential contributions of intestinal microflora (Harrill et al., 2009).

Differences in expression of anti-inflammatory markers such as COX, inducible nitric oxide synthase and interleukins, the rate of formation and conjugation of APAP active metabolite i.e. NAPQI and their excretion through bile and urine are also important indices for species and organ-specific differences in susceptibility to APAP (Gregus et al., 1988; Tee et al., 1987). In addition, it was also shown that alteration in gene expression profile is not only dose- and time-dependent, but may limit the accumulation of potentially toxic products in hepatocytes (Aleksunes et al., 2005).

Conclusion

In conclusion, the limited information provided in this paper show that although COX-2 is expressed in mice kidney tissue, but evidences do not support the

involvement of COX-2 in APAP-induced nephrotoxicity. Likewise, liver injuries due to high dose APAP occurs in absence of COX-2 expression suggesting that APAP hepatotoxicity occurs independent of COX-2 expression.

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Conflict of interest

The authors have no conflict of interest.

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